Simultaneous Determination of Ascorbic and Dehydroascorbic Acids using Newly Developed HILIC Stationary Phases and Tandem Mass Spectrometry

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Expanded Abstract

INTRODUCTION.

- Ascorbic acid (AA) is the first line of defense against reactive oxygen species (ROS) and oxidative stress in plasma (1).
- Changes in the concentration of AA and the ratio of AA to its oxidized form, dehydroascorbic acid (DHAA) are characteristic of chronic inflammatory diseases, such as diabetes (2-4).
- DHAA represents 2-4% of total AA + DHAA in plasma (5) and is generally measured by a subtractive method, either by measuring total AA before and after reduction of DHAA (1, 5), or total DHAA before and after oxidation of AA (6).
- Because of the error inherent in these assays, it is desirable to determine AA and DHAA simultaneously in a single chromatographic analysis.
- In this study we describe an method for simultaneous HILIC negative ion electrospray-tandem mass spectrometry analysis of AA and DHAA.

ANALYTICAL METHOD

- We used a Waters Acquity UPLC interfaced to a Waters Micromass Quattro Premier XE tandem-mass-spectrometer. AA and DHAA were measured by negative ion selective reaction monitoring (SRM). The SRM transitions were 175 to 115 for AA and 173 to 143 for DHAA (Fig. 1).
- We used HILIC as the mode of chromatography in order to enhance the k' and separation of AA and DHAA. In addition, the high level of organic modifier used in HILIC enhances the sensitivity ESI$^-$ -MS/MS.
- To optimize the HILIC mode, we used a new polar polymeric stationery phase (Epic HILIC-POH), developed by ES Industries.

SAMPLES

- Samples of AA or DHAA were dissolved in water containing 100 μM diethyenetriaminepentaacetic acid (DTPA) and stored frozen until analysis. Human plasma was collected in K_3EDTA vacutainer tubes (3), then worked up by precipitation of protein using methanol (6) or by ultrafiltration using a 3,000 MWCO centrifugal ultrafilter (Millipore, Ultrafree). In some cases the samples were applied to C-18 Sep-Pak cartridges (Waters), eluted with water, lyophilized and dissolved in water containing DTPA prior to analysis.
- HILIC chromatography was conducted in isocratic mode. The standard eluant consisted of ammonium formate at various concentrations in 80% acetonitrile. Effects of variation in solvent composition, salt (formate vs. acetate) and salt concentration were evaluated in order to optimize peak shape, resolution, retention time and instrument response.

Results

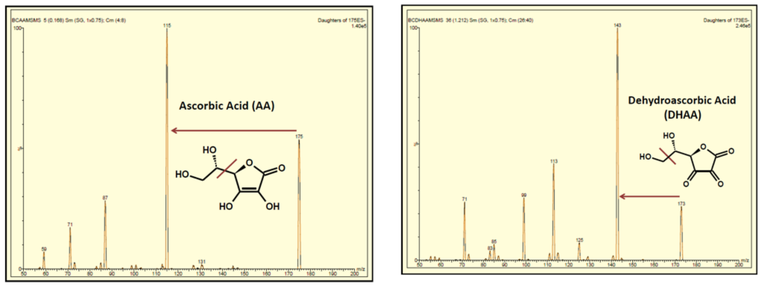

Figure 1. Fragmentation patterns of AA and DHA , showing fragment ions used for negative ion ESI-MS/MS analysis.

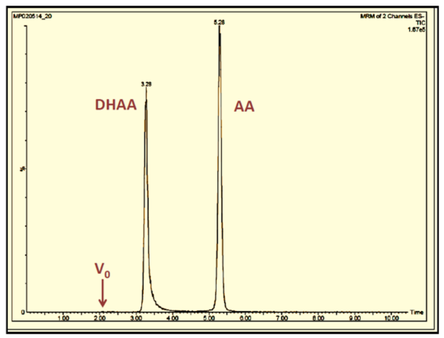

Figure 2. ESI$^-$-MS/MS analysis of a mixture of AA and DHA, conducted on Epic HILIC-POH stationery phase (3 μ, 120 Å pore size, 15 cm x 2.1 mm. Flow rate: 0.2 ml/min. Mobile phase: 80% acetonitrile, containing 20 mM NH_4^+-formate.

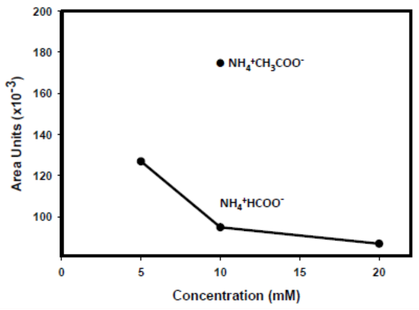

Figure 3. Effect of salt concentration and species on sensitivity for detection of AA. Higher ammonium formate concentrations suppressed signal strength for AA. Acetate, compared to formate, yielded stronger response for AA by negative ion ESI-MS/MS analysis..

Conclusions

1. HILIC chromatography provides good resolution of AA from DHAA, with moderate retention times.
2. The sensitivity for detection for AA and DHAA varied with solvent composition and salt concentration, yielding an ~50% increase in sensitivity between 40 and 5 mM ammonium formate concentration in the mobile phase.
3. Sensitivity for detection of AA and DHAA was improved by use of ammonium acetate vs. ammonium formate in the mobile phase.
4. Using the negative ion electrospray-MS/MS method described above, we were unable to detect a measurable signal for DHAA in plasma samples deproteinized by either methanol precipitation or ultrafiltration.
5. Current efforts are directed at development of a positive ion electrospray MS/MS method for analysis of DHAA in plasma. These assays will also be evaluated using HILIC chromatography.

References

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